Methylated Purines in the Deoxyribonucleic Acid of Various Syrian-Golden-Hamster Tissues after Administration of a Hepatocarcinogenic Dose of Dimethylnitrosamine

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(Received 25 February 1976)

1. DNA was extracted from livers, kidneys and lungs of Syrian golden hamsters at various times (up to 96h) after injection of a hepatocarcinogenic dose of [^{14}C]dimethylnitrosamine. Purine bases were released from the DNA by mild acid hydrolysis and separated by Sephadex G-10 chromatography. 2. At 7h after dimethylnitrosamine administration liver DNA was alkylated to the greatest extent, followed by that of lung and kidney, the values for which were 8 and 3% respectively of those for liver. 3. The O^6 -methylguanine/7-methylguanine ratios were initially the same in all three organs and in the liver DNA of rats under similar conditions of dose. 4. O^6 -Methylguanine was the most persistent alkylated purine in all three hamster tissues. There was evidence for excision of 7-methylguanine, the highest activity for this being present in the liver. 5. Detectable amounts of the minor products 3-methyladenine, 1-methyladenine, 3-methylguanine and 7-methyladenine were present in most hamster tissues, and their individual rates of loss from liver DNA were determined. 6. Ring-labelling of the normal purines in DNA was highest in the liver, followed closely by the lung (80% of that in liver) whereas the kidney had very low incorporation (3% of that in liver). 7. The results are discussed with respect to the hepatotoxicity of dimethylnitrosamine, the miscoding potential of the various alkylation products and the induction of liver tumours in hamsters.

Dimethylnitrosamine is carcinogenic in many animal species, and although a wide variety of organs may be affected, the principal target organ is the liver (see IARC, 1972). In hamsters, single doses or chronic administration of dimethylnitrosamine produce mainly tumours of the liver, and to a lesser extent tumours of the nasal cavity (Tomatis *et al.*, 1964; Herrold, 1967; Tomatis & Cefis, 1967; Haas *et al.*, 1973; Stenback *et al.*, 1973).

The metabolism of dimethylnitrosamine to an alkylating agent, which subsequently reacts with cellular macromolecules, is a prerequisite for the toxic and carcinogenic effects of dimethylnitrosamine, and it has been widely accepted as a working hypothesis that the alkylation of DNA is the critical molecular event in the induction of tumours by dimethylnitrosamine and other alkylating agents (see Magee *et al.*, 1976).

Studies in rats have shown that the initial extent of alkylation of DNA does not correlate with the carcinogenicity of a variety of methylating agents (Swann & Magee, 1968), whereas the persistence of certain alkylated bases in DNA, namely O^6 -methylguanine, may be a determining factor in the tissue-specific induction of tumours (Goth & Rajewsky, 1974; Kleihues & Margison, 1974; Nicoll *et al.*, 1975).

By using a single dose of dimethylnitrosamine which produces a 30% incidence of liver tumours in the hamster (Tomatis & Cefis, 1967), we have investigated the relative stabilities of the alkylated purines in various organs of this species, to examine a possible relationship between the amounts or persistence of the DNA-alkylation products and the organ-specific carcinogenic response.

Experimental

Chemicals

[^{14}C]Dimethylnitrosamine was prepared by Dr. P. F. Swann (Courtauld Institute of Biochemistry, London, U.K.) from [^{14}C]dimethylamine (57 mCi/mmol; The Radiochemical Centre, Amersham, Bucks., U.K.). Before use, it was diluted with unlabelled dimethylnitrosamine (Merck-Schuchardt, Munich, German Federal Republic) to a final specific radioactivity of 3.166 mCi/mmol (hamsters) or 0.74 mCi/mmol (rats).

Animal experiments

Male Syrian golden hamsters (104–136 g), obtained from Animalerie des Essertines, Rochetaillée, France, and male BD-IV rats, bred in this laboratory, were

allowed food pellets and water *ad libitum*, and housed in a room illuminated for 24h per day to minimize the possible effects of diurnal variation in drug-metabolizing activity (see Lake *et al.*, 1976). [^{14}C]-Dimethylnitrosamine was injected intraperitoneally, in 0.9M-NaCl/3mM-sodium citrate, pH 6.0, at a dose of 25mg/kg (hamsters) and 20mg/kg (rats) at about 09.00h. The rats were killed 5h later and groups of three or four hamsters were killed between 7 and 96h later by exsanguination from the abdominal aorta under diethyl ether anaesthesia. Organs were quickly removed, frozen in liquid N_2 and stored at -30°C .

DNA extraction and analysis

DNA was extracted from the pooled organs by a phenol method essentially as described previously (Margison & Kleihues, 1975) and stored in fibrous form at -30°C . Purine bases were released from the DNA by hydrolysis (70°C , 30min) in 1.0ml of 0.1M-HCl per 5mg of DNA (Lawley & Thatcher, 1970). Authentic methylated bases (1-methyladenine, 3-methyladenine, 3-methylguanine, 7-methyladenine, 7-methylguanine or *O*⁶-methylguanine) were added and the solution was adjusted to pH 2.9 by the addition of 0.1M-NaOH, then chromatographed on columns (85cm $\times$ 1cm) of Sephadex G-10 eluted at a flow rate of 15ml/h with 0.05M- NH_3 /0.2% NaN_3 , adjusted to pH 6.75, with formic acid. The eluate was monitored at 250 and 279nm by using a Uvicord III spectrophotometer; 4.5–4.8ml fractions (total 70) were collected, and after those fractions, which according to their elution position and spectral ratio contained guanine and adenine, had been weighed, the E_{260} of each fraction was measured in a Unicam SP.1800 automatic-recording spectrophotometer. When hydrolysates of large quantities of DNA were chromatographed, guanine sometimes precipitated in the peak fractions and could be seen as a crystalline deposit. The addition of 0.1 ml of HClO_4 (70%, w/v) redissolved this precipitate and allowed quantification of guanine.

All fractions except those containing the pyrimidine oligonucleotides were transferred to plastic counting vials and dried in a stream of warm air. The residues were dissolved in 0.3 ml of Hyamine and counted for radioactivity in a Packard 3375 scintillation spectrometer after the addition of 10ml of toluene containing 0.5% diphenyloxazole; quench correction was by automatic external standardization and counting efficiency was around 80%.

Experiments *in vitro*

For the determination of the rates of depurination of the alkylated purines *in vitro*, DNA was isolated from the livers of hamsters killed 7h after injection of

[^{14}C]dimethylnitrosamine. It was dissolved in chloroform-saturated 0.05M-Tris/HCl/0.8M-NaCl, pH 7.2 (2mg/ml), and incubated at 37°C for up to 9 days. Samples (1.0ml) were removed, precipitated with 2vol. of cold ethanol and washed with 70% (v/v) ethanol containing 2% (w/v) sodium acetate until no more radioactivity was detected in the washings. The precipitate was then dehydrated with ethanol, ethanol/diethyl ether (1:1, v/v) and ether and then air-dried. Hydrolysis and chromatography were carried out as described above.

Results

Fig. 1 shows the elution profile from Sephadex G-10 of the purine bases in a 0.1M-HCl hydrolysate of a sample of hamster liver DNA that was isolated 7h after intraperitoneal injection of [^{14}C]dimethylnitrosamine. The elution positions relative to adenine were essentially as reported by Lawley & Shah (1972), except for 3-methyladenine, the elution position of which was found to vary with the pH of the applied sample: at pH 6.0, it was co-eluted with 7-methyladenine, but at pH 2.9 it was eluted between 1-methyladenine and 3-methylguanine.

In preliminary experiments, further analysis of the pyrimidine oligonucleotides have shown the presence of thymine, cytosine, 3-methylcytosine and radioactive material probably derived from phosphotriesters (see O'Connor *et al.*, 1975).

The amounts of 3-methyladenine, 7-methylguanine, *O*⁶-methylguanine and 'metabolic' labelling of guanine and adenine, expressed as a percentage of the parent base, did not usually differ by more than a few per cent when duplicate hydrolysates or DNA preparations were analysed. This was not true for the minor products, 1-methyladenine, 3-methylguanine and 7-methyladenine, which at later times, especially in the lung and kidney (when 3-methyladenine was also affected), could be determined with only moderate accuracy (estimated error 5–15%). This was due to incomplete separation from neighbouring peaks of material containing large quantities of radioactivity and to the extremely small amounts of the minor products themselves. However, at earlier times, these bases were, in most cases, easily determined in all three tissues (see Table 1). In the liver there were sufficient quantities to give a reasonable estimate of their initial rates of loss (see Table 4), but in lung and kidney, the amounts of 1-methyladenine, 3-methylguanine and 7-methyladenine were undetectable after 24h (kidney) or 48h (lung).

3-Methyladenine was also consistently lost from the DNA of all three tissues (except in the kidney, where the maximum content was at 12h), with half-lives of approx. 5–7h (liver) and 12h (lung and kidney). The higher rate of loss from the liver was reflected in the *O*⁶-methylguanine/3-methyladenine

Fig. 1. Sephadex G-10 chromatography of a 0.1 M-HCl hydrolysate of DNA extracted from a sample of the pooled livers of three hamsters killed 7 h after intraperitoneal injection of [¹⁴C]dimethylnitrosamine (25 mg/kg, 3.166 mCi/mmol)

○, Radioactivity; ●, $E_{260}^{1\text{cm}}$. Abbreviations: Py.O, pyrimidine oligonucleotides; 1-MeAde, 1-methyladenine; 3-MeAde, 3-methyladenine; 3-MeGua, 3-methylguanine; 7-MeAde, 7-methyladenine; 7-MeGua, 7-methylguanine; Gua, guanine; Ade, adenine; O⁶-MeGua, O⁶-methylguanine. 3-MeAde, 3-MeGua and O⁶-MeGua were added as markers. For details, see the Experimental section.

Table 1. Alkylated purines in hamster tissue DNA 7 h after administration of [¹⁴C]dimethylnitrosamine

Three hamsters received [¹⁴C]dimethylnitrosamine (25 mg/kg; 3.166 mCi/mmol) and were killed 7 h later. DNA was extracted from the pooled livers, kidneys and lungs and analysed as described in the text.

Product	Tissues ...	Radioactivity (d.p.m./μmol of parent base)		
		Liver	Lung	Kidney
7-Methylguanine		14650	1256	480
O ⁶ -Methylguanine		1900	160	53
3-Methyladenine		570	41	10
7-Methyladenine		415	10	3
1-Methyladenine		176	2	1
3-Methylguanine		65	6	—

ratio: this was about 2.5 in all tissues at 7 h but increased to 13.5 (lung), 18.5 (kidney) and 45 (liver) by 36 h.

The amounts of the major methylation products

7-methylguanine and O⁶-methylguanine in liver, lung and kidney DNA between 7 and 96 h after [¹⁴C]dimethylnitrosamine injection is shown in Fig. 2. In liver and lung during the first 36 h 7-methylguanine was lost consistently, whereas the amount of O⁶-methylguanine was constant. Between 36 and 48 h, there was a sudden decrease in both 7-methylguanine and O⁶-methylguanine, and afterwards 7-methylguanine was lost at about the pre-36 h rate and O⁶-methylguanine was lost slowly. In the kidney, alkylation seemed to be incomplete until about 12 h and the 'step' in the decay curves occurred between 24 and 36 h.

The relative amounts of base alkylation (O⁶-methylguanine/7-methylguanine or O⁶-methylguanine/3-methyladenine) compensates first for effects of toxicity, which would influence the loss of these alkylation products to similar extents, and secondly for differences in the actual dose of dimethylnitrosamine given (and, to some extent, its rate of metabolism), which would affect the initial extent of alkylation. The almost linear increase in the O⁶-methylguanine/7-methylguanine ratio in liver, lung and kidney with time (Fig. 3) indicates that the irregu-

Fig. 2. Amounts of the major alkylpurines in hamster tissue DNA at various times after intraperitoneal injection of [¹⁴C]dimethylnitrosamine (25 mg/kg, 3.166 mCi/mmol)

Open symbols and dashed line, O⁶-MeGua; closed symbols and continuous line, 7-MeGua; ○, ●, liver; □, ■, lung; △, ▲, kidney.

larities in the decay curves (Fig. 2) probably truly reflect the events *in vivo*, i.e. are due to biological variation, and are not a consequence of the extraction and analysis of the DNA.

The higher rate of loss of 7-methylguanine compared with O⁶-methylguanine was most marked in the liver, in which the ratio at 96h was six times that at 7h. The amounts in lung and kidney DNA increased in parallel up to 48h and by 96h the ratios were 3.5 times (lung) and 3.3 times (kidney) the 7h values. For comparison, the change in the O⁶-methylguanine/7-methylguanine ratio in DNA incubated *in vitro* is given (Fig. 3). In this experiment, DNA was isolated from the livers of hamsters killed 7h after injection of [¹⁴C]dimethylnitrosamine and incubated for various times *in vitro* at 37°C and pH 7.2. The labile alkyl purines were continuously released, and under the conditions used, 7-methylguanine was lost with a half-life of 192h and 3-methyladenine with a half-life of about 18h. O⁶-Methylguanine remained bound to the DNA throughout the period studied. There were insufficient amounts of the minor products 1-methyladenine, 3-methylguanine or 7-methyladenine to give accurate rates of loss; however, 7-methyladenine was no longer detectable by 48h.

The amount of 7-methylguanine in the DNA of lung and kidney relative to that in liver DNA increased during the course of the experiment *in vivo*,

Fig. 3. Relative amounts of 7-methylguanine and O⁶-methylguanine in hamster tissue DNA at various times after intraperitoneal injection of [¹⁴C]dimethylnitrosamine (25 mg/kg, 3.166 mCi/mmol)

●, Liver; ■, lung; ▲, kidney. ○---○, Increase in the O⁶-methylguanine/7-methylguanine ratio during incubation *in vitro* of DNA that had been isolated from the liver 7h after injection of [¹⁴C]dimethylnitrosamine (25 mg/kg). For details see the Experimental section.

showing that 7-methylguanine was lost more rapidly from liver than from lung or kidney DNA (Table 2). There was a general trend for the amount of O⁶-methylguanine in lung to increase relative to that in liver, indicating a more rapid loss of this base from liver (Table 2). However, this must be confirmed by determination of alkylated bases at later intervals after dimethylnitrosamine administration. This also applies to the results for the kidney, the relative amounts of O⁶-methylguanine being too dispersed to indicate whether loss of this product was more or less rapid than from the liver.

The extent of incorporation of radioactivity into guanine in the DNA of liver, lung and kidney during 12–96h after injection of [¹⁴C]dimethylnitrosamine is shown in Fig. 4. The guanine/adenine ratio for metabolic incorporation was always 0.6–0.7. Although incorporation generally increased, this was not so between 36 and 48h in the liver and lung or between 24 and 36h in the kidney, which corresponded (in time) with the sudden decrease in the major alkylated bases (see Fig. 2).

When rats and hamsters were given approximately the same dose of dimethylnitrosamine and killed

Table 2. Relative amounts of 7-methylguanine and O⁶-methylguanine in hamster tissue DNA at various times after administration of [¹⁴C]dimethylnitrosamine

Groups of three or four hamsters received [¹⁴C]dimethylnitrosamine (25 mg/kg, 3.166 mCi/mmol) and were killed after the times indicated. DNA was extracted from the pooled livers, lungs and kidneys and analysed as described in the Experimental section. Results are expressed as a percentage of the amount of the base present in liver DNA, the value of which is given in parentheses (as d.p.m./μmol of guanine).

Time after administration (h)	7-Methylguanine			O ⁶ -Methylguanine		
	Liver	Lung	Kidney	Liver	Lung	Kidney
7	100 (14650)	8.6	3.3	100 (1900)	8.4	2.8
12	100 (12200)	9.6	7.2	100 (1900)	9.1	6.6
24	100 (10100)	9.0	7.3	100 (2160)	7.5	5.7
36	100 (6320)	14.0	6.5	100 (1960)	10.1	4.4
48	100 (2690)	18.1	13.0	100 (1117)	11.2	7.6
96	100 (1100)	24.6	15.1	100 (935)	13.2	6.5

Fig. 4. Incorporation of radioactivity into guanine in DNA of hamster tissues at various times after intraperitoneal injection of [¹⁴C]dimethylnitrosamine (25 mg/kg, 3.166 mCi/mmol)

After Sephadex G-10 chromatography of 0.1M-HCl hydrolysates of DNA, the amount of guanine and the radioactivity co-eluted with it were determined as described in the Experimental section. ○, Liver; □, lung; △, kidney.

about the same time afterwards, the extent of alkylation in hamster liver DNA was 1.5 times that in rat liver DNA, but the O⁶-methylguanine/7-methylguanine ratios were almost the same (Table 3).

Discussion

The alkylation of DNA is strongly suspected to be the critical initiating step in the induction of tumours by alkylating agents. Such a reaction could be considered a self-sufficient prerequisite for transformation, but cellular, organ or body responses may be the final determining factors in the development of a tumour. In the somatic-mutation theory of carcinogenesis, the consequences of a reaction with DNA would be expected to lead to mutational changes and to result in a cell with an altered genotype.

The nature of the reaction of alkylating agents with DNA has been studied in attempts to discover whether there is a direct relationship between DNA alkylation and carcinogenesis. Various approaches have produced a great deal of information about the activation of certain indirect agents, the mechanisms of reaction of various agents with DNA and the spectrum of reaction products and their relative stabilities (see Magee *et al.*, 1976; Lawley, 1976).

It has thus been found that all alkylating agents react with DNA bases mainly at the 7-position of guanine, but the extent of this reaction in the DNA of rat tissues does not correspond to the carcinogenicity of a series of methylating or ethylating agents (Swann & Magee, 1968, 1971; Goth & Rajewsky, 1972; Kleihues & Magee, 1973). On the other hand, large differences have been found in the amounts of some of the minor products, especially the O-alkylation products, and this can be explained on the basis of the alkylating mechanism (see Lawley, 1974). One of these minor products, O⁶-methylguanine, was suggested to be potentially mutagenic (Loveless, 1969), since methylation at the O⁶-position could block base pairing with cytidine and cause misincorporation of thymine (see Lawley, 1974). This miscoding potential was subsequently demonstrated in experiments *in vitro* using a RNA-dependent RNA polymerase and a template containing O⁶-methylguanine,

Table 3. Alkylation of rat and hamster liver nucleic acids *in vivo* and *in vitro* by [¹⁴C]dimethylnitrosamine

DNA, extracted from the pooled livers of two rats or three hamsters, which were killed 5 or 7 h after injection of 20 or 25 mg of [¹⁴C]dimethylnitrosamine/kg was analysed as described in the Experimental section. Slices of livers from normal rats or hamsters were incubated with [¹⁴C]dimethylnitrosamine *in vitro* and the amount of 7-methylguanine in total nucleic acids was determined. Alkylation was expressed as the percentage conversion of the guanine present into 7-methylguanine (*in vivo* and *in vitro*) or as O⁶-methylguanine/7-methylguanine ratio (*in vivo*).

Species	<i>In vivo</i>		<i>In vitro</i> *
	7-Methylguanine (mol/100mol of guanine)	O ⁶ -/7-Methylguanine (ratio)	7-Methylguanine (mol/100mol of guanine)
BD-IV rat	0.284	0.142	0.19
Syrian golden hamster	0.440	0.130	0.29

* Taken from Montesano & Magee (1974).

Table 4. Loss of minor alkylated purines from hamster liver DNA

For the results *in vivo*, groups of three or four hamsters received [¹⁴C]dimethylnitrosamine (25 mg/kg, 3.166 mCi/mmol) and were killed at various times later. DNA was extracted from the pooled livers and analysed as described in the Experimental section. The half-lives *in vitro* were taken from Lawley (1976) (indicated by *) or determined by incubation of DNA extracted from the livers of animals killed 7 h after injection of [¹⁴C]dimethylnitrosamine (see the Experimental section). Enzymic excision for the products was considered likely or unlikely according to the difference between the half-lives *in vivo* and *in vitro* (see the text).

Product	Half-life (h)		Enzymic excision
	<i>in vivo</i>	<i>in vitro</i>	
3-Methyladenine	5	18	Likely
1-Methyladenine	4	Stable	Likely
7-Methyladenine	5.5	3.3*	Unlikely
3-Methylguanine	14	>110*	Likely

which misincorporated the thymine analogue uracil into the product (Gerchman & Ludlum, 1973).

Although 3-methylcytosine has also been shown to cause mispairing in the test system *in vitro* (Ludlum & Wilhelm, 1968; Ludlum, 1970, 1971; Singer & Fraenkel-Conrat, 1970), the quantities of this base that are produced by 'weak' alkylating carcinogens are greater than by 'strong' carcinogens (see Lawley, 1974). However, the reverse is true for O⁶-methylguanine, and recently much attention has been given to this product.

An enzyme that excised O⁶-methylguanine from rat liver DNA *in vivo* was suggested as a possible factor in the tissue-specific effect of certain chemical carcinogens (O'Connor *et al.*, 1973). Further, the lack or very low extent of such an activity in rat brain compared with other rat tissues provided an explana-

tion for the specific induction of neurogenic tumours by alkylnitrosoureas (Goth & Rajewsky, 1974; Kleihues & Margison, 1974; Margison & Kleihues, 1975). Also, the greater persistence of O⁶-methylguanine in the DNA of rat kidney compared with rat liver after a single high dose of dimethylnitrosamine was correlated with the induction of kidney but not liver tumours under these conditions (Nicoll *et al.*, 1975).

In the present experiments, the induction of tumours by dimethylnitrosamine is considered with respect to DNA alkylation in the Syrian golden hamster, both as an individual species and in comparison with the rat. The amounts of the methylated purines in DNA have been determined in the liver, lung and kidney of hamsters various times after administration of [¹⁴C]dimethylnitrosamine to assess whether there is a direct relationship between alkylation and tumour induction and whether these tissues are capable of active excision of methylation products.

The dose (25 mg/kg) of dimethylnitrosamine applied would result in approximately a 30% incidence of liver tumours in the surviving hamsters (Tomatis & Cefis, 1967). In comparison, no tumours would be induced in the liver of normal rats receiving the same dose (Hilfrich *et al.*, 1975) even though the amounts of the major methylation products (7-methylguanine and O⁶-methylguanine) in hamster liver DNA were initially only 1.5 times higher than in rat liver DNA (see Table 3). (This value corresponds very closely to the ratio of liver nucleic acid methylation in hamster to that in rat after incubation of tissue slices with [¹⁴C]dimethylnitrosamine *in vitro*.) There is thus no correlation between the initial amounts of 7-methylguanine or O⁶-methylguanine in liver DNA and the relative incidence of liver tumours in these two species.

In the hamster, the maximum alkylation occurs in the liver, which is the principal target organ, followed by the lung and then the kidney (Table 1). Similarly, in rats, liver DNA had the highest extent of alkylation

but that in kidney was more extensively alkylated than that in lung (Swann & Magee, 1968).

As indicated above, the lack of induction of liver tumours by single doses of dimethylnitrosamine in the normal rat has been attributed to the high capacity of rat liver to enzymically excise *O*⁶-methylguanine from its DNA. In contrast, hamster tissues have an extremely low capacity for excision of *O*⁶-methylguanine, and although the liver was more active than lung in this process (see Table 2), throughout the experimental period the liver maintained the highest content of this 'promutagenic' base, but relatively lower at 96h than at 7h (see Fig. 2).

Fig. 2 shows that the amounts of the major alkylated bases fell suddenly between 36 and 48h in liver and lung DNA, and between 24 and 36h in kidney DNA. At the same times, the extent of ring-labelling of normal purines in DNA (which were derived from C₁ breakdown products of [¹⁴C]dimethylnitrosamine; see Magee *et al.*, 1976) showed similar decreases (Fig. 4). Since the ratio of the major products increased consistently in all tissues (Fig. 3), these effects were probably due to the toxicity of dimethylnitrosamine, which resulted in either the degradation of the DNA in heavily damaged cells or a wave of regenerative DNA synthesis (presumably by using salvage pathways), or to a combination of both.

The hepatotoxicity of dimethylnitrosamine may be an important event in tumour induction in hamster liver, since regenerative DNA synthesis would take place on a template containing many potentially miscoding lesions. In rats, on the other hand, regenerative DNA synthesis starts in the liver at about 24h after a hepatotoxic dose of dimethylnitrosamine (Stewart & Magee, 1971), when the content of *O*⁶-methylguanine has been much decreased by excision. If, however, dimethylnitrosamine (or *N*-methyl-*N*-nitrosourea) is applied to rats during the course of DNA synthesis after partial hepatectomy or carbon tetrachloride administration, liver tumours are induced (Craddock, 1971; Craddock & Frei, 1974; Pound *et al.*, 1973). Evidence for very extensive necrosis in hamster liver was obtained histologically (G. P. Margison & R. Montesano, unpublished work). However, information is needed on this and the extent and time of DNA synthesis in various hamster tissues after dimethylnitrosamine application in order to discover to what extent these processes might contribute to the tissue specificity of this agent.

The increase in the *O*⁶-methylguanine/7-methylguanine ratio in liver DNA compared with that from lung, kidney or DNA incubated *in vitro* (Fig. 3), a comparison of the half-lives of 7-methylguanine *in vitro* (8 days) and *in vivo* (26h) and the decrease in the relative amounts of 7-methylguanine in the three organs (Table 2) cannot be explained by toxic effects and suggest that 7-methylguanine may be excised by an active process. However, this cannot be corroborated

because of an incomplete knowledge of the ionic environment of DNA *in vivo* (Margison *et al.*, 1973). Active excision of 7-methylguanine has also been suggested to take place during chronic administration of dimethylnitrosamine to mice (Nemoto & Takayama, 1974). These results are in contrast with those in the rat, in which 7-methylguanine appears to be lost from the DNA of those tissues so far studied at a rate that is relatively much closer to the rate of depurination *in vitro* and an active excision mechanism seems not to be implicated (Craddock, 1969; Kleihues & Margison, 1974).

This problem also applies to the data for the labile minor alkylated purines, which are summarized in Table 4. The influence of pH and ionic environment on the depurination of labile bases *in vitro* has been partially determined only for 7-methylguanine and 3-methyladenine (Margison *et al.*, 1973; Margison & O'Connor, 1973), but extrapolation from these data and comparison of the rates of loss *in vivo* and *in vitro* suggest that 3-methyladenine and 3-methylguanine are actively excised whereas 7-methyladenine is not. On the other hand, 1-methyladenine is a chemically stable product and its loss *in vivo* clearly indicates active excision. Similar conclusions were reached for 3-methyladenine in rat liver (Margison & O'Connor, 1973), but the very small amounts of the other minor methylation products have so far made stability studies *in vivo* impractical. It is tempting to suggest that the active removal of these minor products is a consequence of their possible miscoding potential. However, this has not yet been demonstrated *in vitro* and no conclusions can be made.

The loss of some of the minor alkylation products suggests that specific products are excised by specific enzymes which, as might be the case with *O*⁶-methylguanine, become saturated when high concentrations of the substrate are present. Experiments using very low doses of the direct alkylating agent *N*-[³H]methyl-*N*-nitrosourea have indeed shown that hamster liver is able to excise *O*⁶-methylguanine rapidly when only small amounts are produced in DNA (G. P. Margison, J. M. Margison & R. Montesano, unpublished work). It has also been shown that in rat kidney the rate of excision of *O*⁶-methylguanine from DNA is dependent on the dose of dimethylnitrosamine administered (Nicol *et al.*, 1975).

The present results are compatible with the hypothesis that *O*⁶-alkylguanine in DNA is an important factor in the induction of tumours by alkylating agents, since the DNA in the liver, which is the target organ, maintained the highest content of this product throughout the experimental period.

This work was supported by NCI Contract no. 1CP-55630. We thank Miss Pauline Stafford-Smith and Miss Susan Bassill for secretarial assistance.

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